EXPERIMENTS ON THE DEVELOPMENT OF THE GILLS IN THE AMPHIBIAN EMBRYO.[1]

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The present work upon the gills was undertaken as a continuation of the study of relations of symmetry in embryonic organs, in the hope that it might throw light on questions raised in previous studies of the limbs.[2] The external gills of the salamander were chosen to this end, as constituting another system with right and left enantiomorphs, each without any plane, axis, or center of symmetry in itself. Owing to the complexity of the system, however, if for no other reason, the experiments failed to give as clear evidence on the questions raised as the limbs had done. Nevertheless, they have brought to light certain interesting facts, which in part corroborate and in part extend those reported by Ekman ('13), who worked upon various anuran embryos. They show, furthermore, certain differences between *Amblystoma* and the anurans with respect to the organs studied.

The gill arches and external gills form a complex in which all of the germ layers are represented. There is an outer covering of ectoderm, a central core of mesoderm, which contains the blood vessels and extends out into the gill filaments, and an inner lining to the arches and the clefts consisting of endoderm. Besides these the ganglion crest contributes elements to the arches, chiefly to the formation of the cartilaginous skeleton.

The experimental work of Ekman upon anurans has shown the following: (1) That when the ectoderm covering the gill region is removed, the regenerated ectoderm is capable of forming gill filaments. (2) That when the gill ectoderm is replaced by ectoderm from another region, with certain exceptions, the for-

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[2] Harrison ('17 and '21).

mation of gills is suppressed. (3) That the external gills and the outer branchial grooves are specifically determined in the ectoderm and that the endoderm plays no part in the localization of the gills. (4) That the ectodermal rudiments of the gills have, from early stages on, a certain power of self-differentiation which varies amongst the several species studied. (5) That gills which arise from displaced ectoderm, together with atypical endoderm, conform entirely as to their position, form, and size, to the ectoderm. (6) That the branchial blood vessels are an essential factor in the later development of the gills.

The present experiments were made upon the embryo of a urodele, *Amblystoma punctatum*. They concern almost entirely the two outer layers, ectoderm and mesoderm. Owing to technical difficulties, the endoderm was experimented with only to a very limited extent, and this layer, therefore, does not figure in the present analysis. While certain things are quite definite, the work must be considered as incomplete, inasmuch as there has been no opportunity as yet to examine the specimens in sections.

With regard to the technique of operation, only one thing requires special mention. The donor embryo was in most cases stained *intra vitam* in Nile blue sulphate, which colors the ectoderm bright blue.[3] The stain persists for many days, so that the exact delimitation of the graft can be observed, as development proceeds. This removes a serious source of error, for it is thus always possible to determine whether the parts under observation are derived from the host or the graft. Embryos from stage 21 (Fig. 1, medullary folds just closed) to stage 25 (just before appearance of tail bud[4]) were used for nearly all the experiments. A few were made with earlier and a few with slightly later stages. Subsequent experiments made with still later stages show that marked changes in the behavior of the ectoderm after transplantation occur at about the time when the mesectoderm from the ganglion crest grows down and the first branchial pouch is developed (stage 29). These will not be considered at present.

[3] Detwiler ('17).

[4] Stages 25 and 29 have been figured previously (Harrison '18, Figs. 1 and 2).

Removal of the Ectoderm of the Branchial Region and its Replacement by Ectoderm from Another Part of the Body.

The gill region in an embryo with closed medullary folds is shown in outline in Fig. 1 (*BR*). If this ectoderm is removed and replaced by ectoderm from the flank (*FL*) or any other part except that immediately surrounding the gill region, complete suppression of the operculum and the gills on the operated side (Fig. 2) results. Thus, out of twenty-four cases in which ectoderm from the flank was used, seventeen had the gills entirely lacking. In six cases in which the third gill developed and in one in which the first gill was formed, ectoderm from the host covered the greater part of the gill including the tip.

When the covering ectoderm is taken from the region just behind the gills, *i.e.*, from the pronephric and limb area (*PN*), functional gills develop. This was found in five cases, in three of which all three gills were well developed, while in the two others the first gill was either absent or rudimentary.

When, however, the ectoderm is taken from the ventral mid-region (*CD*), *i.e.*, from the region immediately ventral to the branchial, the result is not so favorable. Out of nine cases, no gills at all developed in four, a functional third gill in one, a functional third and rudimentary second in three, and two functional gills (second and third) in one.

Likewise, when the covering is taken from the anterior part of the head, there is usually a partial development of the gills, involving principally the second and third. The transplanted piece was taken in various ways (Fig. 1, *C*, *C′*, *A*). In general, it may be said that the closer the seat of origin of the graft is to the normal branchial region, the more perfect are the gills that develop. A supernumerary balancer developed in almost all of the cases in which the graft included the normal balancer ectoderm.

When the ectoderm covering the gill region is removed and the wound left uncovered, as it was in the donor embryos in many experiments, the surrounding ectoderm pushes over the wound, covering it usually in twenty-four hours or a little more.

The mortality among these embryos is high, but when they live, gills nearly always develop from this regenerated ectoderm. In some cases the gills formed are normal; in others, there is a reduction in number or in size or both, the first gill being more often affected than the others.

Bilateral Extirpation of Branchial Ectoderm.

As far as the question of the morphology of the gills is concerned, this experiment is in no wise different from the unilateral operations, but physiologically it may prove of greater interest. At present only a few cases are available for study. In three experiments the branchial ectoderm was removed on both sides and replaced by ectoderm from the flank. In two of these cases no gills at all developed, while in one the first gill on the left side, which was partly covered by ectoderm from the host, was rudimentary. In the last-named case the circulation of the blood was never established and the embryo became œdematous. In both of the other cases the circulation was established in the vessels of the yolk, but the heart action became more and more feeble, and after a time the circulation of the blood ceased entirely. Both of these eventually became œdematous also, though not extremely so. One individual, killed fourteen days after the operation, showed marked lack of sensitiveness to tactile stimuli, and responded to a needle prick only with a single jerk. It was apparently unable to swim. The other could execute swimming movements fairly well, though in a somewhat jerky fashion. A fourth case that should be considered in this connection had the branchial ectoderm removed on both sides after which it was allowed to regenerate without replacement from elsewhere. The operculum was formed on both sides but the gills failed to develop except in most rudimentary form—on the right side two rudimentary gills and on the left only a sharp conical process (Fig. 3). Blood circulating in these gills was first observed thirteen days after the operation. The larva was kept under observation for thirty-six days, at which age it was preserved. Its development, apart from the absence of gills, was normal.

The experiments are too few in number to warrant very definite conclusions. Absence of gills from both sides seems, in some cases at least, to lead to weakened heart action and ultimate stoppage of the circulation. On the other hand, the last case shows that it is possible for the embryo to adjust itself to the almost complete loss of its respiratory organs, sufficient aeration of the blood probably taking place through the general surface of the body.

Change of Orientation of the Branchial Ectoderm.

These experiments were for the purpose of determining whether there is a definite polarization of the ectodermal elements, and, further, whether any specific gill pattern is predetermined in the region. The experiments of the preceding sections already indicate that the latter is not the case.

Transplantations were made in four ways, as was done in the case of the experiments with limbs. The ectoderm was removed from the gill region and replaced by similar ectoderm either from the same or from the opposite side of the body and placed either with the dorsal or with the ventral border of the graft corresponding to the dorsal border of the wound. Besides these a few experiments were made in which the graft was rotated only 90°. The results may be stated very simply, for there is a marked tendency toward normal development in all combinations. Forty-eight experiments were made, twenty-eight of which gave definite results. Thirteen yielded normal gill-complexes, and these were distributed amongst all of the six different orientations tried. In five cases the second and third gills were normal but the first was small; in two cases the first gill was rudimentary while the others were normal; and in three cases the first gill was altogether lacking, the other two being normal. There were only three cases in which the gill-complex was irregular.

From these experiments the conclusion may be drawn that the branchial ectoderm is equipotential, and that there is no polarization of the elements affecting the potencies of differentiation. In this respect the branchial ectoderm of *Amblystoma* is markedly different from that of the anurans studied by Ekman.

In the latter changes in orientation of this layer are followed by corresponding changes in the position of the gills. This lack of axial differentiation in the gill ectoderm of the *Amblystoma* embryo lasts, however, for a brief period only, for in slightly later stages (from stage 29 on) change in orientation of the ectoderm is followed by marked disturbances in the arrangement of the gills.

Removal of the Mesoderm of the Branchial Region.

No satisfactory method of removing the mesoderm of the gill region and replacing it by other mesoderm has been worked out. However, it is a simple matter to remove all of this layer from the region in question and to cover the wound with clean ectoderm from the branchial region of another embryo. This affords the surrounding mesoderm an opportunity to wander in and fill the place taken by the original branchial mesoderm. The wound after such operations heals, though not nearly so readily as when there is mesoderm underlying the outer layer.

Sixteen such experiments were made, of which ten were successful. In only two of these cases was there any radical disturbance of the normal development of the gills. The other eight individuals developed approximately normally, though in five of them the heart was affected and the circulation was never established in the embryo. In three cases, however, the circulation was established on both sides, the gills of the operated side remaining smaller than the normal ones. In one the first gill was suppressed.

From the fact that in such a large proportion of cases the development of the circulation was radically interfered with, it seems likely that the removal of the mesoderm must have disturbed the material that goes to make up the heart. Aside from this the effect of the operation is slight. The mesoderm of the surrounding region obviously has the potency to fill in the gap made by the removal of tissue and then to take part in the development of normally formed, though at first smaller, gills.

Change of Orientation of the Branchial Ectoderm and Mesoderm.

These experiments are similar to those in which the ectoderm alone was turned, differing only in the circumstance that both ectoderm and mesoderm were lifted and implanted together. There is no difficulty in separating the mesoderm from the endoderm, though the healing of the wounds after transplantation is much less regular than when the ectoderm alone is taken. Inasmuch as turning the ectoderm alone has no effect upon the development of the gills, the effect of the transplantation of the two layers together must be due to the mesoderm alone. The respective results are strikingly different after the two operations.

There were sixty-three experiments in which both mesoderm and ectoderm were transplanted. Thirty-seven were positive. These cases were distributed unevenly amongst the different orientations, but the results throughout, except when the graft was from the same side of the body and normally oriented, were extremely variable and difficult to place in distinct categories.

The normally oriented grafts (homopleural dorsodorsal) gave five normal or nearly normal cases and one distinctly abnormal individual in which two gills were irregular and fused together.

In none of the other orientations were there any absolutely normal cases. However, in the inverted grafts from the opposite side of the body (heteropleural dorsoventral) there were three approximately normal individuals out of eleven positive experiments. Of the remaining eight cases, no two have been given the same designation except two which have been classified as very irregular and two others put down as rudimentary. Three gills were distinguishable in five of the eight cases, and only one gill was present in one. In one of the eight cases, and possibly in others, the abnormalities consisted in defects which might have been due to general conditions rather than to any particular disturbance of orientation.

Inverted grafts from the same side of the body (homopleural dorsoventral) gave not a single case, in a total of thirteen, that even approached the normal condition. Two gills were de-

veloped in five cases, but they were abnormal in some respects (Fig. 4); in the others only one gill was formed. Two individuals had quite irregular gills and three had rudimentary ones.

In the non-inverted grafts from the opposite side of the body (heteropleural dorsodorsal), of which there were only six available cases, the results were in no two cases exactly alike. In one a nearly normal complex developed. In two others three gills developed, but with abnormalities. The others were still less perfect.

The above results, standing alone, do not lead to any definite conclusion regarding the exact rôle of the mesoderm in the formation of the gills or regarding the nature of the disturbance caused by abnormal orientation of this layer. It is not clear that it is due simply to the reversal of a single polarized axis, such as is the case in the mesoderm of the anterior limb, although the fact that there is a certain tendency toward normal development in the inverted grafts from the opposite side of the body might be taken to indicate that the anteroposterior axis of the elements of this layer is more markedly polarized than is the dorsoventral. The experiments described in the next section point somewhat more clearly to such a conclusion.

Effect of Increasing the Amount of Mesoderm in the Branchial Region. Superposed Grafts.

In this group of experiments the branchial ectoderm was removed without materially injuring the underlying mesoderm, and a piece of tissue of the same size and shape including both mesoderm and ectoderm was healed over the wound. In this way the amount of mesoderm was approximately doubled, there having been only a very slight loss of mesoderm cells through the operation. The grafts were oriented in the four different ways as in the previous experiments.

The group with normal orientation (homopleural dorsodorsal) developed gills that were normal or nearly so in seven cases (Fig. 5), and in only one, in which the three gills were closely fused, was there any marked abnormality.

The inverted grafts from the opposite side of the body (hetero-

pleural dorsoventral) formed in all five cases gills which approached the normal condition. In two of these the gills were distinctly smaller than normal and in the other three there was considerable fusion and some irregularity, though all three gills could be distinguished.

On the other hand, the inverted grafts from the same side of the body (homopleural dorsoventral) showed in all five cases a very defective development of the gills, only one small, irregular or abortive gill being formed in each case.

In the non-inverted grafts from the opposite side of the body (heteropleural dorsodorsal) the results were likewise for the most part irregular or defective. Two fused gills developed in two cases, and in the other three only a single small, irregular or abortive gill was formed.

The results of these experiments show that while the doubling of the mesodermal material has in itself little or no morphogenetic effect, the orientation of the superadded material is of considerable consequence in determining whether aberrations in the development of the gills occur or not. Aside from experiments in which the transplanted material is normally oriented, these disturbances are apparently least marked in the case of inverted grafts from the opposite side of the body, and in this respect there is an approach to the condition found in the mesoderm of the fore limb bud. However, the results of this orientation of the grafted gill mesoderm are not normal with sufficient constancy to term the combination "harmonic", as was done in the case of the limb.

Removal of both Ectoderm and Mesoderm Without Replacement of Either.

This experiment is a by-product of the experiments described in the last two sections. The individuals are the donor embryos of those operations.

With such a large deep wound as is necessary in removing the gill mesoderm, a high mortality was to have been expected. Out of one hundred and six operations, only ten cases survived ten days or longer. Five others were preserved at intervals from an age of two to that of eight days.

The regeneration that takes place after this operation is very imperfect. In four cases a single fairly normal but small gill was regenerated, identified as the first gill in two cases, the second in one and the third in one. In three of these there were, in addition, one or two rudimentary gills. In the other cases either rudimentary gills alone were formed, or else no regeneration of gills took place at all. Blood circulation was established on the operated side in only two individuals. The circulation was defective throughout the embryo in four cases, as shown by the œdema that developed.

The imperfect restitution that takes place in these experiments must be ascribed to the severity of the operation rather than to the removal of anything specifically essential, for when either the mesoderm or the ectoderm alone is removed there is often complete restoration of function and structure.

Transplantation of Branchial Ectoderm.

The branchial ectoderm was transplanted either to the flank or to the anterior part of the head in a few cases, in order to test the potency of this tissue in strange surroundings. In all four individuals of the first group the results were essentially the same. Small nodules, which might possibly be interpreted as incipient gill sprouts, developed, but after a few days they became obliterated. In one case of the second group, where the gill ectoderm was transplanted to cover the eye and mandibular region, no supernumerary gills were formed and even the normal first gill failed to develop. On the other hand in two cases in which the graft was implanted not quite so far forward (Fig. 1, *A*) there were traces of rudimentary gills in the region of the hyoid and mandibular arches respectively. One of these (Fig. 6) showed two supernumerary filaments quite distinctly, in which, however, the circulation failed to become established. In two other cases, operated in a similar way, no supernumerary filaments were observed.

The potency of the gill ectoderm to form gills in any of these abnormal positions is obviously far less marked in *Amblystoma* than in the anuran species studied by Ekman, especially in *Rana fusca*.

Transplantation of Branchial Ectoderm and Mesoderm.

The results of this experiment are scarcely more definite than when the branchial ectoderm alone is transplanted. There is an initiation of development but it soon becomes arrested.

In five cases the whole of the branchial mound, including both ectoderm and mesoderm but no endoderm, was placed directly behind the normal gills, *i.e.*, in the region of or just ventral to the pronephros, the ectoderm and mesoderm having been previously removed. About the fourth or fifth day a marked prominence was present in the region of the graft, and, in two of the cases at least, distinct gill sprouts showed. There was, however, no furthei development; the grafted tissue gradually flattened out and became reduced to a slight hump or nodule.

In three other cases the graft was placed in front of the normal gills, in place of the material normally constituting the mandibular and hyoid arches. The results were not very different, except that the original gills of the embryo were to some extent disturbed in their development. No gills ever developed in front of the normal ones and in all three cases the balancer was suppressed.

Effect of Lack of Function on the Development of Gills.

It often occurs in experiments with amphibian embryos that the circulatory system does not function properly. Sometimes no movement of the blood can be observed even though the heart does beat. The effect of this upon the gills is noticeable; they never expand fully, and, while preserving their essential character, they have an atrophic appearance.

In experiments on the gills in particular it not infrequently happens that, although the embryo is otherwise normal, the circulation in the operated gills is either delayed or fails altogether to become established. The same atrophic appearance is found in such cases.

When the heart is removed a similar condition ensues as regards the gills, but the effects on the embryo as a whole are much more pronounced. It becomes badly swollen and ulti-

mately dies. Operations of this kind were done upon embryos in stages 33 and 34, *i.e.*, just before the heart begins to beat. It is difficult to remove the heart rudiment completely. There is almost always some regeneration, and pulsation is established, which, however, is usually ineffective and in such cases no blood can be seen in movement in the yolk vessels or through the gills. Nevertheless, the latter grow out, and are normally constituted except for their atrophic appearance. The operculum and the gill plates are also formed. As regards the development of their specific form, the gills, like many other structures, are thus independent of function, though the marks of atrophy in the functionless organs are unmistakable.

Conclusion and Summary.

So far as the present experimental analysis has led, the respective rôles of ectoderm and mesoderm in the formation of the gills are as follows:

The branchial ectoderm is specifically gill forming, but surrounding the gill region, and, more particularly, posterior to it, the ectoderm has the potency to form gills in a diminishing intensity as the distance from the gills increases. This is shown both by transplantation experiments and by regeneration after removal of the branchial ectoderm.

The gill pattern is not, however, laid down in the branchial ectoderm, as is shown by the fact that this same ectoderm, when turned in any way or even when taken from the opposite side, gives rise to normal gills notwithstanding.

The specific pattern must therefore be determined by the deeper layers, but in the absence of experiments upon the endoderm, the exact rôles of mesoderm and endoderm, respectively, cannot be determined. The indications are, however, that the endoderm does not play merely a passive part, as Ekman maintains to be the case in the anurans.

The mesoderm cannot be a well-defined mosaic, for doubling of the material by superposition, provided the orientation of the grafted tissue is normal, does not disturb the normal development of the gills. Abnormal orientation of the mesoderm, however,

both after extirpation of the normal mesoderm and in cases of superposition, results in marked aberration from the normal development, showing that this layer is not without axial differentiation. These aberrations from the normal are not specific for any particular orientation, but there is a greater degree of disturbance after the orientations which, in the case of the fore limb, have been found to be disharmonic, *i.e.*, inverted mesoderm from the same side of the head and non-inverted from the opposite side. One of the combinations called harmonic in the case of the fore limb (that in which the graft is normally oriented) gives normal gills, but the other, while yielding a considerable number of cases of approximately normal gills, results on the other hand in a good many irregularities. These circumstances may be taken to indicate that the gill-mesoderm elements are distinctly polarized in an anteroposterior direction and perhaps less markedly so dorsoventrally. The fact that the mesoderm from the periphery of the gill region is capable of forming gills, taken together with the fact that doubling the amount of the material does not disturb normal development, points to the conclusion that this mesoderm is equipotent.

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EXPLANATION OF PLATE I.

Figure 1. Profile view of an embryo of *Amblystoma punctatum*, stage 21. The various flaps of ectoderm used in the transplantations are indicated by ovals. A, includes the mandibular, hyoid and anterior part of the branchial region; BR, branchial region; C, ventroanterior part of head region; C′, head region; CD, cardiac region; Fl, flank; PN, pronephric and limb region. $\times$ 10.

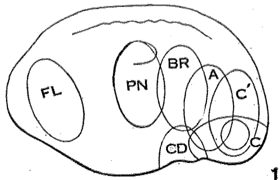
Figure 2. Replacement of branchial ectoderm by ectoderm from the flank (Exp. E. Br. E. 21) resulting in complete suppression of gills on the right side; ventral view of larva eighteen days after operation. $\times$ 10.

Figure 3. Larva with gills almost suppressed through failure to regenerate after removal of branchial ectoderm (Exp. E. Br. M 15–); specimen preserved thirty-six days after operation. $\times$ 10.

Figure 4. Larva with abnormal gills on the right side due to inversion of the branchial ectoderm and mesoderm (Exp. Br 35), specimen preserved fifteen days after operation. $\times$ 10.

Figure 5. Individual showing normal gills developed from double quantity of mesoderm (Exp. S. Br. 17); specimen preserved ten days after operation. $\times$ 10.

Figure 6. Individual in which branchial ectoderm (Fig. 1, BR) had been transplanted to a position just anterior to gill region (Fig. 1, A) (Exp. Br. A 2); two supernumerary gills (H and M) developed; the dotted line indicates the boundary of the transplanted ectoderm; sketch made ten days after operation. $\times$ 10.



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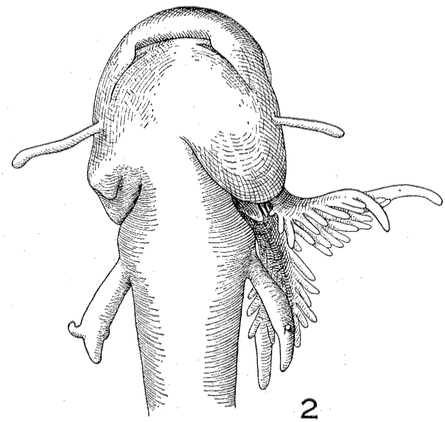

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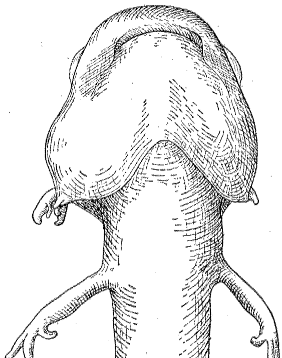

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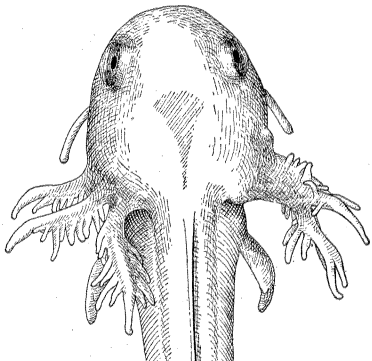

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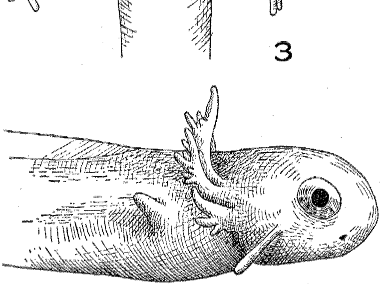

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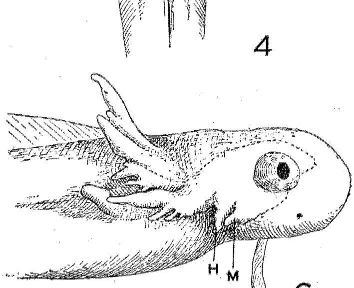

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